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Title Page

Frequent hepatocyte chimerism in long-term human liver allografts independent of graft outcome

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Abstract

Microchimerism after liver transplantation is considered to promote graft tolerance or tissue repair, but its significance is controversial. By using multiplex polymerase chain reaction (PCR) of short tandem repeat (STR) loci after laser capture microdissection of hepatocyte nuclei, we compared the proportions of recipient-derived hepatocytes in long-term stable liver allografts and late dysfunctional allografts caused by chronic rejection or idiopathic post-transplantation hepatitis. Through fluorescence *in situ* hybridization (FISH), we also analyzed the presence of recipient-derived Y-positive hepatocytes in the biopsies of livers transplanted from female donors to male recipients. The study population comprised 24 pediatric liver transplant recipients who survived with the initial graft, whose 10-year protocol biopsy records were available, and who had normal liver function (stable graft, SG; $n = 13$) or a late dysfunctional graft (LDG; $n = 11$) with similar follow-up periods (mean 10.8 years in the SG group and 11.2 years in the LDG group). STR analysis revealed that hepatocyte chimerism occurred in 7 of 13 (54%) SGs and 5 of 11 (45%) LDGs ($p = 0.68$). The proportion of hepatocyte chimerism was low, with a mean of 3% seen in 2 of 3 female-to-male transplanted livers (one each of SG and LDG).

In conclusion, hepatocyte chimerism was a constant event. The extent of engraftment of recipient-derived hepatocytes does not seem to correlate with the degree of hepatic injury in long-term liver allografts.

Keywords

pediatric liver transplantation, tolerance, STR-PCR, laser capture microdissection,

microchimerism

Footnote

ALT, alanine aminotransferase; AST, aspartate aminotransferase; CR, chronic rejection; FISH, fluorescence *in situ* hybridization; FFPE, formalin-fixed, paraffin-embedded; G-CSF, granulocyte colony-stimulating factor; IPTH, idiopathic post-transplantation hepatitis; LDG, late dysfunctional graft; PCR, polymerase chain reaction; SG, stable graft; STR, short tandem repeat; T-Bil, total bilirubin

1. Introduction

In contrast to other transplanted organs, the liver has been shown to be capable of inducing tolerance. The presence of chimerism may be one explanation for how the liver induces tolerance [1]. Lagaaij et al. [2] have demonstrated how the replacement of donor endothelial cells by those of a recipient is correlated with vascular rejection in renal transplantation. In liver transplantation, the progressive engraftment by the recipient's inflammatory cells (i.e., Kupffer cells) soon after transplantation has been well described [3-7]. Meanwhile, Pons et al. [8] reported that endothelial cell chimerism does not influence allograft tolerance in liver transplant patients, and Tanaka et al. [9] observed that endothelial cell chimerism is a time-dependent event after liver transplantation independent of graft dysfunction. A few studies have demonstrated human intragraft chimerism in hepatocytes [4,7,10-14]. Previous reports have shown that hepatocyte chimerism occurs early after transplantation, from 1 week to as late as 63 months after surgery, and that the proportion of patients in whom this has occurred has been low [4,12,14]. The significance of hepatocyte chimerism to transplant outcome has not yet been determined.

In cases of sex mismatch between the graft and the recipient, the identification of the Y chromosome using *in situ* hybridization is widely used to study intragraft chimerism [3,5-8,10]. However, this approach has limitations because it is only applicable to cases of sex-mismatched grafts, and the Y chromosome is not always detectable, even in males. The *in situ* hybridization procedure holds the potential of causing damage to antigens, leading to a low sensitivity [6,7,10]. On the other hand, there is a less damaging procedure that uses short tandem repeats (STR) consisting of highly

polymorphic tetranucleotide repeat sequences that are distributed throughout the genome. The use of these markers for the assessment of chimerism has the advantage of being independent from sex mismatch, and this technique requires only small samples [15]. An approach combining laser capture microdissection of target cells with subsequent polymerase chain reaction (PCR) analyzing highly polymorphic STR markers to detect hepatocytes of recipient origin enabled us to investigate a great number of liver tissue samples without limitations [11]. There have been only two reports detecting hepatocyte chimerism in liver allografts using this method [4,11]. The technique in one of the studies was to manually microdissect the liver acini so that contamination of the hepatocyte samples by blood cells could not be avoided [4].

The consequences of the phenomenon of microchimerism are still unknown. Microchimerism may promote graft tolerance or participate in tissue repair after epithelial damage [16]. The chimeric cells are thought to be derived from recipient stem cells. The mechanism of microchimerism, whether transdifferentiation [10,17,18] or cell fusion [19-21], is unknown. Because of the possibility of hepatocyte chimerism in long-term grafts, which may have an influence on graft stability, we analyzed the presence of recipient-derived hepatocytes in liver biopsies by a multiplex STR typing kit. We compared the proportions of recipient-derived hepatocytes between long-term stable grafts and dysfunctional grafts (i.e., having chronic rejection) of similar follow-up periods to examine whether microchimerism exerts any influence on the long-term fate of grafts. Fluorescence *in situ* hybridization (FISH) for X and Y chromosomes was also performed in 3 of 24 cases with female-to-male transplantation.

2. Objective

The aim of this study was to use STR-based genotyping to analyze donor hepatocyte replacement by recipient cells in liver allografts in relation to the long-term outcome (stable graft or chronic rejection) after liver transplantation. For quantitative evaluation of the degree of microchimerism, FISH for X and Y chromosomes was used additionally in cases of female-to-male transplantation.

3. Material and Methods

Patients

This retrospective study reviewed the files of the Department of Diagnostic Pathology of Kyoto University Hospital, and selected, from among 117 pediatric liver transplant patients, those who received liver allografts from living donors and who underwent their last biopsies at 10 years post-transplant from August 2006 to August 2008. All subjects were ≤ 18 years of age at the time of liver transplantation and had received liver grafts from their parents.

The study subjects were divided into two groups. The first group consisted of 13 patients who survived with the initial grafts, who had available records on their 10-year protocol biopsies, and whose liver function tests were normal (stable graft (SG) group). The patients in this group showed no histological signs of rejection in the absence of immunosuppression or a low maintenance dose of tacrolimus at the time of their last biopsy. The second group consisted of 11 patients who survived with the initial grafts, but who experienced graft dysfunction after a follow-up period similar to the stable group (late dysfunctional graft (LDG) group). The diagnoses of graft dysfunction were chronic rejection (CR, $n = 3$) and idiopathic post-transplantation hepatitis (IPTH, $n = 8$). The demographic data of the recipients and donors are shown in Table 1.

This study was approved by the Kyoto University Review Board (No. G504).

Definition of late graft dysfunction and histological assessments

Histological analysis was performed on formalin-fixed paraffin-embedded tissue samples. Morphological findings were assessed using hematoxylin and eosin staining (H&E), Masson's trichrome staining, and immunohistochemical staining for cytokeratin

7 of the bile duct epithelium. Liver biopsy specimens were assessed by pathologists (AM-H, and HH). Chronic rejection (CR) can be defined as immune-mediated damage to the liver allograft, which is characterized histologically by two main features: loss of small bile ducts and an obliterative vasculopathy that affects large- and medium-sized arteries [22]. Idiopathic post-transplantation hepatitis (IPTH) can be defined as chronic hepatitis after liver transplantation for which the causes are unknown. IPTH responds to steroids, but if untreated, the condition could progress to cirrhosis and graft failure [23]. IPTH and late acute rejection are likely to be parts of an overlapping spectrum of immune-mediated damage [24].

Postoperative clinical data were collected retrospectively. Laboratory data at the time of protocol biopsies for stable patients and at the time of diagnosis of post-transplant CR and IPTH included the following variables: serum aspartate aminotransferase (AST, normal range, 13-29 IU/l), alanine aminotransferase (ALT, 8-28 IU/l) and total bilirubin (T-Bil, 0.2-1.0 mg/dl).

Laser capture microdissection and DNA extraction

To identify donor and recipient alleles, DNA from the explanted recipient native livers and normal liver samples obtained from each donor liver at transplantation was analyzed without microdissection. For the post-transplant liver allograft biopsy specimens, laser capture microdissection of hepatocyte nuclei was performed using the PALM Laser-MicroBeam system (P.A.L.M, Wolfratshausen, Germany). Only the nuclei of hepatocytes were microdissected in order to avoid blood cells. Paraffin sections (4 μ m thick) were deparaffinized and lightly stained with toluidine blue. At least 1000

hepatocyte nuclei were dissected from stable grafts ($n = 13$) and 11 samples of late graft dysfunction (Figure 1). The laser spot size was set in 6 μm , the size of hepatocyte nuclei [25]. After collecting the cells onto the lid of a tube, the DNA was isolated using the QIAamp DNA FFPE Tissue Kit (QIAGEN, Venlo, the Netherlands).

Short tandem repeat (STR) PCR

Three liver tissue samples for each case, including the recipient's explanted native liver, the donor liver (pre-implantation liver tissue) and the long-term-followed allograft were used for detecting graft chimerism. We used the AmpFlSTR® Identifiler® PCR Amplification Kit (Life Technologies, Carlsbad, CA, USA) to perform STR-PCR. The 15 STR loci amplified in this reaction included: D8S1179, D21S11, D7S820, CSF1PO, D3S1358, TH01, D13S317, D16S539, D2S1338, D19S433, vWA, TPOX, D18S51, D5S818, and FGA. PCR was performed using 1 ng of genomic DNA in a final reaction volume of 25 μl . The PCR cycle conditions were: 95°C for 11 min, followed by 31 cycles at 94°C for 1 min, 59°C for 1 min and 72°C for 1 min. The final elongation step was 45 min at 60°C. The PCR products were analyzed with an ABI PRISM® 310 Genetic Analyzer (Life Technologies, Carlsbad, CA, USA).

In situ hybridization for X and Y chromosomes

After proteinase K treatment, FISH was performed on pretreated slides for the X chromosome (chromosome enumeration probe [CEP] X, spectrum green) and Y chromosome (CEP Y, spectrum red) (Abbott Molecular, Inc., Des Plaines, IL, USA). The slides were denatured for 10 min at 73°C and hybridized overnight at 37°C in an

incubation chamber. DAPI (4',6-diamidino-2-phenylindole) was used for nuclear counterstaining (Vysis, Inc., Downers Grove, IL, USA). The slides were analyzed under fluorescence microscopy at a magnification of 600 \times . We focused on hepatocytes, which were recognized via their morphology and location within the plates. Grafts from female-to-female transplantation or male-to-male transplantation were stained as controls. The percentage of hepatocyte chimerism was calculated by counting 100 hepatocytes for each slide for sex-mismatched cases; in particular, chimeric hepatocytes can be identified by the presence of a Y chromosome signal in female-to-male liver transplantation. Because a proportion of polyploid hepatocytes is normally present in the liver [26], an XX signal in male grafts transplanted to female patients could not be counted as recipient-derived cells as it might be a sectioning artifact [25].

Statistical analysis

Quantitative data were expressed as mean \pm SD. Student's t-test was used to compare continuous variables. The chi-square test or Fisher's exact test was used to compare frequencies of categorical variables. Statistical analysis was performed using StataSE 9.0 (Stata Corporation, College Station, TX, USA). A p-value less than 0.05 was considered significant.

4. Results

Laboratory and histological findings

The first group, the SG group, was composed of 13 patients who exhibited normal liver function test results after a long-term follow-up period (10.8 ± 2.2 years). The causes for liver transplantation in this group were biliary atresia in 12 patients and congenital biliary dilatation in one patient. Ten of the patients received left or lateral grafts from their mother, and 3 received grafts from their father. Patients with SGs received either a low maintenance dose of tacrolimus monotherapy (trough level <1.5 ng/ml), or had the tacrolimus discontinued at the time of the last protocol biopsy. There were minimal histological abnormalities, including slight perivenular fibrosis and non-specific mild inflammatory changes (Figure 2A). No vascular or biliary complications were found in any of the SG patients.

The second group, the LDG group, was composed of 8 patients with IPTH (Figure 2B) and 3 with CR (Figure 2C). The original diseases in this group were congenital biliary atresia in 10 patients and Wilson's disease in one patient. The patients survived with their initial grafts, but exhibited abnormal liver function test results after a follow-up period similar to the SG group (11.2 ± 2.4 years). Six of the LDG patients received left or lateral grafts from their mother, and 5 received grafts from their father.

There were significant differences in the liver function test results and age in recipients and donors between the SG and LDG groups at 10 years post-transplant ($p < 0.05$) (Table 1). All patients in the LDG group were treated with a triple immunosuppression regimen of tacrolimus, prednisolone, and mycophenolate mofetil.

STR-PCR after laser microdissection

Although normal women with a previous male pregnancy can have a Y chromosome in their liver [29,30], none of the female donor livers revealed maternal chimerism in our study.

A total of 72 samples from 24 recipients (13 recipients with stable grafts, and 11 recipients with graft dysfunction), taken from each recipient's native liver, donor liver and allograft at 10 years post-transplant, were evaluated with Identifiler® STR kits (Life Technologies). Allele peaks detected allografts at 10 years, and were compared with the recipient and donor samples to determine if the amplified alleles originated from the recipient or donor or represented a mixture of both. There was a loss of signal on the longer-sized STR products, probably due to highly degraded and fragmented DNA molecules on formalin-fixed paraffin-embedded sections. Since all donors were the parents of the recipients, at least one of the alleles was shared between donor and recipient. When the recipient and donor had two distinct alleles for a given marker (heterozygosity), the appearance of one allele from the recipient not shared between the donor and recipient at any STR loci indicated hepatocyte chimerism in the allograft (Figure 3A). When two of the STR alleles were shared between donor and recipient, the marker was not informative for chimerism; therefore, the STR loci were not counted as informative signals. As a result, the mean number of informative STR loci showing signals was 2.0 ± 1.3 in 24 allografts. Hepatocyte chimerism was found in 7 of 13 cases (54%) in the SG group and 5 of 11 cases (45%) in the LDG group ($p = 0.68$). The STR loci that exhibited recipients' alleles in the allografts are shown in Table 2. The STR loci that provided shorter amplified PCR products less than 250 bp long, such as D3S1358,

D5S818, D8S1179, D19S433 and vWA, allowed a clear discrimination between recipient and donor. One case showed a recipient allele for the longer fragment locus CSF1PO (281-317 bp long).

XY chromosomes *in situ* hybridization

After laser microdissection and STR-PCR analysis, 3 of 24 recipient liver biopsy samples were obtained from male recipients receiving grafts from female donors; two in the SG group and one in the LDG group. The quantitative evaluation revealed one patient in the SG group and one in the LDG group who showed that 3.4% (6/175 hepatocytes) of hepatocytes and 2.5% (3/120) of hepatocytes were XY-configured hepatocytes, respectively (Figure 3B). Thus, the proportion of hepatocyte chimerism was low in both groups [25]. One case in the SG group revealed no evidence of chimerism on STR-PCR analysis, but recipient-derived hepatocytes were detected on XY-FISH.

5. Discussion

We confirmed that hepatocyte chimerism was present at high frequencies in human liver allografts in our study subjects. In a previous report by Kleeberger [11], hepatocyte chimerism was reported to be present in a high percentage as analyzed by laser microdissection followed by microsatellite analysis; i.e., 41% in the 27 post-transplantation liver biopsies. A study by Ng et al. [4] found 80% in the 10 post-transplantation liver biopsies.

The biological significance of chimeric cells is controversial. Hepatocyte chimerism seems to be triggered by extensive liver cell turnover as it occurs in chronic active hepatitis reported by Kleeberger [11]. However, in our study, we found that the degree of hepatocyte chimerism observed long after liver transplantation was not different in SG and LDG patients and did not seem to depend on the degree of hepatic injury. Ng et al. [4] found that only up to 1% of recipient-derived cells show hepatocyte differentiation, and that most liver allografts have only mild nonspecific changes. In addition, the severity of acute cellular rejection appears to have no effect on the rate of recipient-derived repopulation [12].

Obtaining DNA for molecular analysis from formalin-fixed, paraffin-embedded (FFPE) tissue is a challenge. When fixing is performed too long, it can cause damage to nucleic acids by extensive cross-links between proteins in the tissues and DNA fragmentation. DNA from FFPE tissue is often scarce, degraded, and can contain substances that inhibit the molecular procedures, leading to low quality DNA. FFPE tissues stored for long periods have shown a lower rate of amplification in the PCR reaction than that of recent FFPE samples [27]. The standard protocol of the AmpFlSTR® Identifiler® PCR

Amplification Kit can produce results with DNA templates of more than 100 pg, approximately 16 diploid cells [28]. For FFPE tissue samples, even if a large number of cells is contained, highly degraded DNA may result in poor PCR amplification [27]. Our FFPE samples were not uniform with respect to time of fixation and storage time of the blocks in each case, which could explain the low PCR amplification rate and the variation of PCR amplification products and frequent loss of signals in our study. Quantitation of STR PCRs may not be feasible for highly degraded FFPE samples; thus, positivity for multiple recipient-specific loci (compared to one) in the graft may not represent a higher extent of chimerism. Even if 1,000 dissected nuclei are contaminated by a few blood cells, this minor contamination in the DNA fragment will not be amplified [28].

One case in the present study displayed hepatocyte chimerism on FISH, but no evidence of chimerism in STR-PCR. Generally, FISH methodologies underestimate the extent of chimerism because of sectioning and/or suboptimal hybridization efficiency [6,7,10]. Because of highly degraded DNA from FFPE tissue, the case in the present study had only one STR locus that was informative for chimerism. Including the result of FISH, 8 recipients in the SG group and 5 recipients in the LDG group showed hepatocyte chimerism; these findings were not statistically significant ($p = 0.43$).

It is yet unknown whether recipient-derived hepatocytes originate either by adult progenitor-cell transdifferentiation or by fusion between stem cells and donor hepatocytes [31]. Adult bone marrow-derived stem cells have been capable of differentiating into hepatic cells in the rat [17] and the human [10,32], but the level of

hepatocyte replacement after bone marrow transplantation has been reported to be quite low [33]. Five percent of recipient-derived hepatocytes become detectable only after granulocyte colony-stimulating factor (G-CSF) treatment post-liver transplantation, suggesting that stem cell mobilization initiates microchimerism in hepatocytes [31]. G-CSF treatment or bone marrow cell infusion in patients with chronic liver disease leads to improvement of liver function, which suggests the contribution of engrafted chimeric cells in liver regeneration [34-36]. However, in our study, we found that the extent of engraftment of recipient-derived hepatocytes was very small in both the SG and LDG groups, comprising 3% of counted hepatocytes. The degree of liver cell chimerism did not seem to influence graft outcome. Eleven of the 16 samples obtained from recipients with sex-mismatched grafts demonstrated recipient-derived hepatocyte repopulation, comprising a mean of 2.1% of the hepatocytes, similar to our result [12]. We concluded that the chimerism was a constant event, and that liver injury was not necessary to achieve hepatocyte replacement by bone marrow-derived cells [33]. This result was in agreement with the report in renal transplantation in which tubular epithelium replacement by recipient cells is observed at low percentages of tubular epithelial cells (2.4% to 6.6%) and is not correlated to outcome [37].

6. Conclusions

In the present study, STR-based genotyping after laser microdissection revealed a high percentage of chimeric hepatocytes in allografts long after liver transplantation. No correlation was found with allograft outcome. Evaluation of the Y chromosomes by *in situ* hybridization showed low percentages of chimeric hepatocytes.

Figure legends

Figure 1.

Laser microdissection of hepatocyte nuclei. Sequence of pictures showing dissection of a hepatocyte by laser microdissection. The laser spot size is set in 6 μm , the size of hepatocyte nuclei (middle figure). The hepatocyte nuclei are dissected selectively and catapulted into the lid of the tube (lower figure).

Figure 2. Histological findings of stable graft (A) and late dysfunctional graft (B and C).

(A) Stable graft showing no remarkable change (HE, original magnification, 200 \times).

(B) Idiopathic post-transplant chronic hepatitis showing bridging fibrosis with mild interface change (HE, original magnification, 200 \times).

(C) Atrophy of portal tract and loss of bile ducts in chronic rejection. No recognizable interlobular bile duct on CK7 immunostaining (original magnification, 200 \times). A, hepatic artery; P, portal vein.

Figure 3. Hepatocyte microchimerism in long-term allograft.

(A) Chimeric allotype in long-term hepatocyte allograft post-transplant by short-tandem repeat PCR.

(B) Fluorescence *in situ* hybridization for X (green) and Y (red) chromosomes. Y chromosome signal (red) identified on hepatocyte nuclei in female-to-male transplantation.

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Table 1: Clinical characteristics of the study population

	Patients with stable grafts (n = 13)	Patients with late dysfunctional grafts (n = 11)	p values
Age at LT (mean \pm SD) (yr)	1.2 \pm 1.3	6.7 \pm 5.3	0.002*
Recipient gender (female)	10 (77%)	9 (82%)	0.77
Time since LT (yr)	10.8 \pm 2.3	11.2 \pm 2.5	0.69
Donor age (yr)	31 \pm 3.9	36 \pm 5.7	0.01*
Donor gender (female)	10 (77%)	6 (55%)	0.25
AST (IU/l)	28 \pm 4.8	126 \pm 76	<0.001*
ALT (IU/l)	23 \pm 7.4	156 \pm 197	0.02*
T-Bil (mg/dl)	0.9 \pm 0.3	4.7 \pm 6.9	0.06

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; BA, biliary atresia; LT, liver transplantation; T-Bil, total bilirubin; yr, year

*p<0.05

Table 2: The result of hepatocyte chimerism analyzed by microsatellite analysis and FISH for X and Y chromosomes

Case no.	Graft dysfunction	Recipient age (years)	D-R	% Y+ hepatocytes in F-M transplantation	STR loci showing chimerism	Number of informative STR loci (locus name)
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Stable graft group

1	SG	0	F-F		—	1 (D19)
2	SG	0	F-F		D3	3(D3, D5, D8)
3	SG	0	F-M	3.4% (6 XY/175 cells)	—	1 (D3)
4	SG	0	F-F		D19	1 (D19)
5	SG	1	F-F		D7, D18, CSF	3(D7, D18, CSF)
6	SG	1	F-F		—	3(D3, D7, D19)
7	SG	1	M-F		D3	1 (D3)
8	SG	1	F-F		—	1 (TH)
9	SG	1	F-F		D8	1 (D8)
10	SG	1	F-M	0% (0 XY/100 cells)	—	6 (D3, D5, D8, D19, vWA, TPO)
11	SG	3	F-F		D3, D8, D19, vWA	4 (D3, D8, D19, vWA)
12	SG	3	M-F		—	3 (D3, D8, TH)
13	SG	4	M-M		D3, D5	3 (D3, D5, vWA)

Late dysfunctional graft group

14	IPTH	1	F-F		—	1 (TH)
15	IPTH	2	F-M	2.5% (3 XY/121 cells)	D3, D8	4 (D3, D5, D8, D19)
16	CR	2	F-F		D19	2 (D3, D19)
17	IPTH	4	M-F		—	1 (D3)

18	CR	5	F-F		—	1 (D3)
19	IPTH	6	F-F		D19	1 (D19)
20	IPTH	6	M-M		—	1 (D3)
21	IPTH	7	M-F		D16	1 (D16)
22	IPTH	8	M-F		—	2 (D3, D5)
23	CR	15	M-F		D3, D8	2 (D3, D8)
24	IPTH	18	F-F		—	1 (D13)

Abbreviations: CR, chronic rejection; D-R, donor-recipient; F, female; FISH, fluorescence in situ hybridization; IPTH, idiopathic post-transplantation hepatitis; M, male; SG, stable graft; STR, short tandem repeat

D3, D3S1358; D5, D5S818; D7, D7S820; D8, D8S1179; D13, D13S317; D16, D16S539; D18, D18S51; D19, D19D433; CSF, CSF1PO; TH, TH01; TPO, TPOX

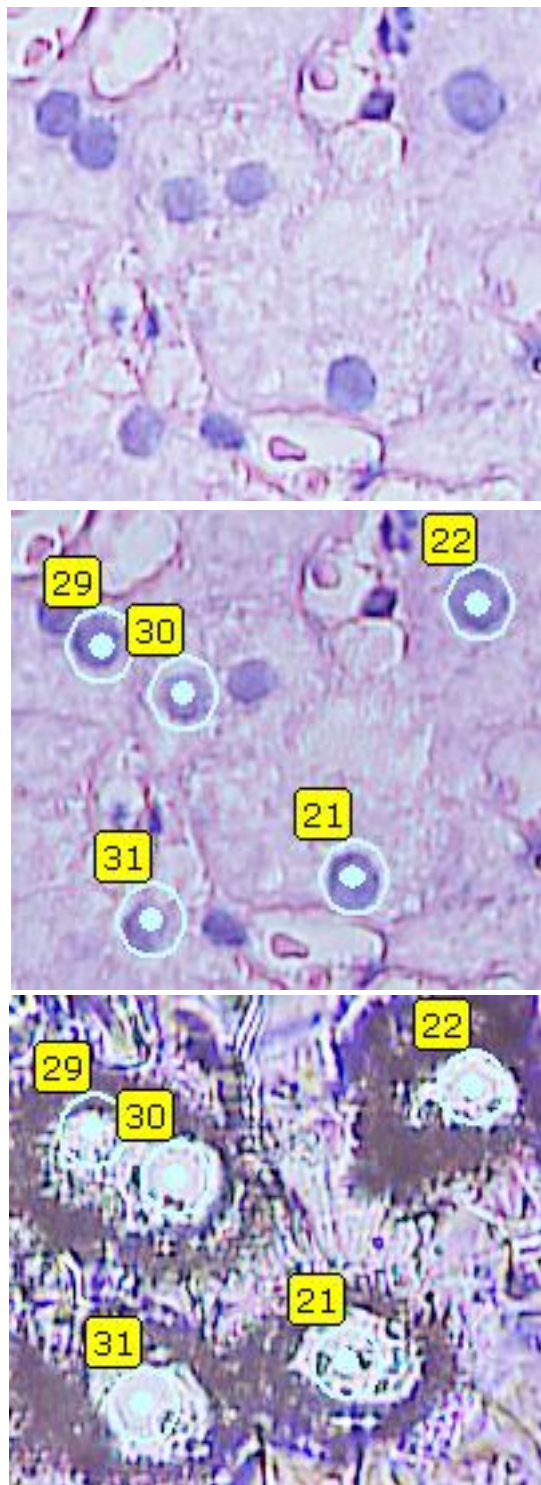


Figure 1

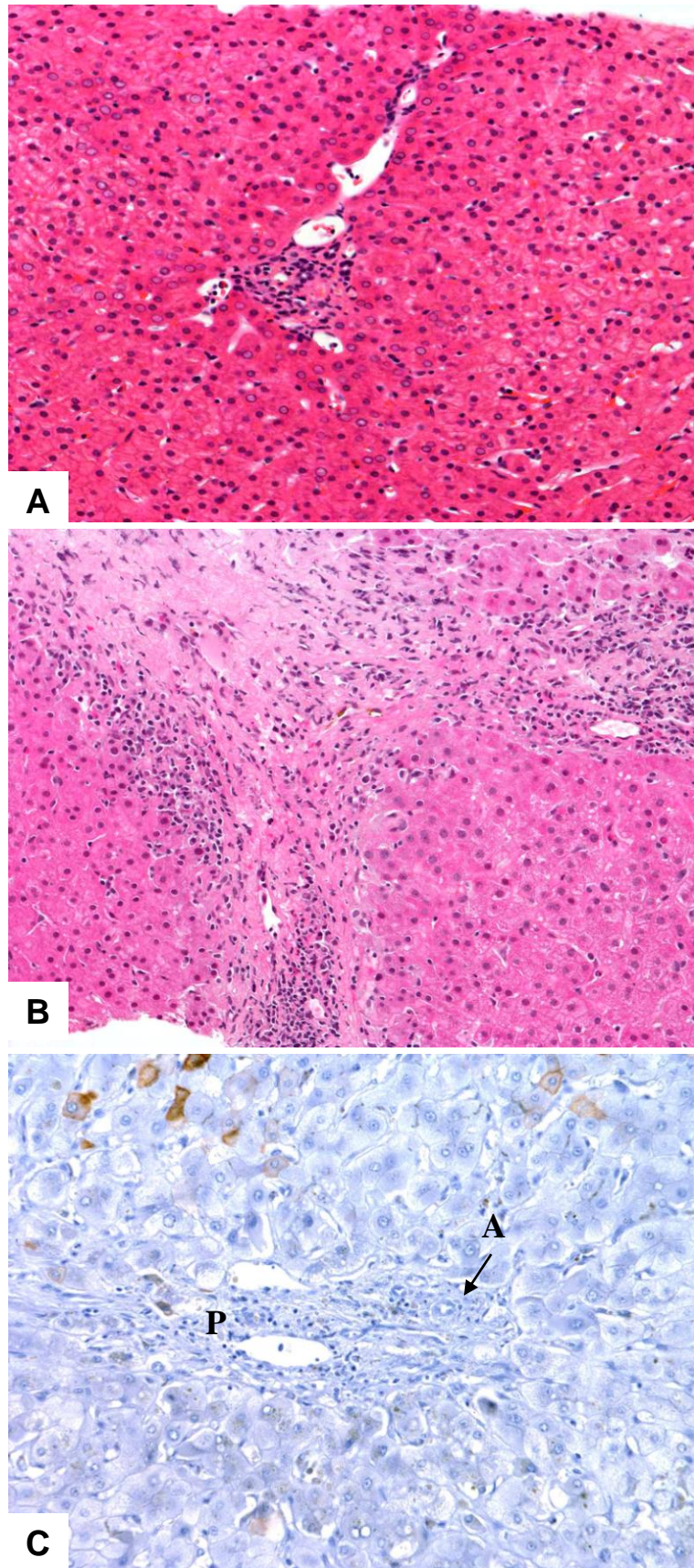
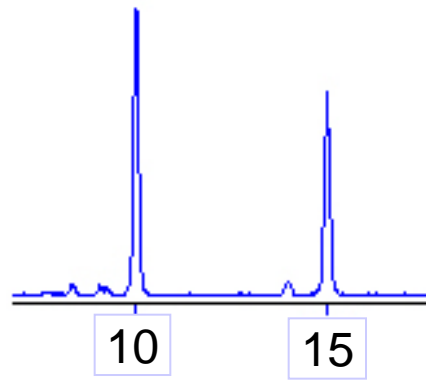


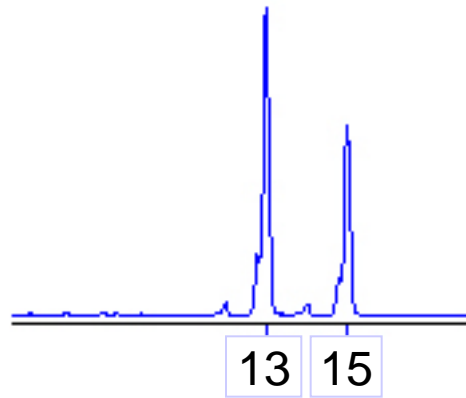
Figure 2

A

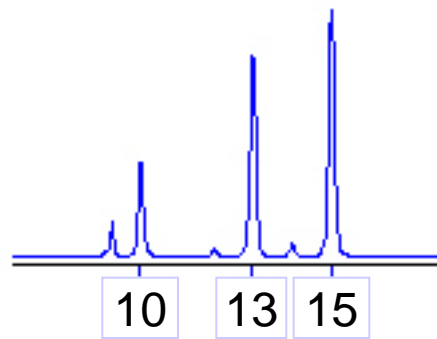
D8S1179



Recipient



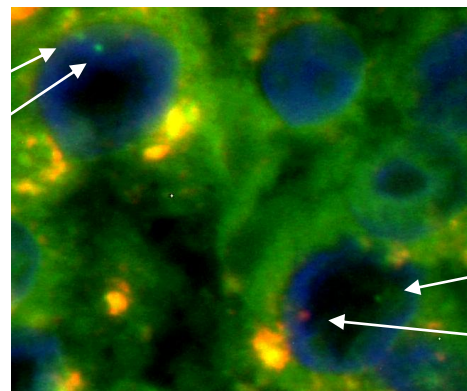
Donor



Allograft

B

X
X



X
Y

Figure 3